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SHORT COMMUNICATION

Evidence for a role of β-1,3-glucanase in dicot seed germination

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Summary

Class I β-1,3-glucanases are antifungal vacuolar proteins implicated in plant defense that show developmental, hormonal, and pathogenesis-related regulation. The expression was studied in germinating tobacco seeds of a chimeric β-glucuronidase (GUS) reporter gene fused to 1.6 kb of the 5' flanking sequence of the tobacco class I β-1,3-glucanase B (GLB) promoter. Histological staining for GUS activity showed that expression of the GLB promoter is highly localized in a specific zone of the endosperm in germinating seeds. The temporal and spatial patterns of GUS and β-1,3-glucanase activity found, suggest a novel function for class I β-1,3-glucanases during seed germination in a dicotyledonous plant.

Introduction

Endo-β-1,3-glucanases (EC 3.2.1.39) are abundant, highly regulated hydrolytic enzymes found in all higher plants examined (reviewed by Meins *et al.*, 1992). Although there is indirect evidence implicating β-1,3-glucanases in the defense of plants against fungal infection (reviewed by Boller, 1988), apart from a specialized function in pollen development (Worrall *et al.*, 1992), their endogenous function during plant growth and development is not known.

Class I β-1,3-glucanase isoforms of tobacco, which are the subject of our study, are basic, vacuolar proteins that are expressed in an organ- and cell-type-specific manner (reviewed by Meins *et al.*, 1992). They accumulate at high concentrations in the roots and in the epidermis of lower leaves, but not in young leaves near the top of the plant (Felix and Meins, 1986; Memelink *et al.*, 1990; Neale *et al.*, 1990). Accumulation of β-1,3-glucanases depends on the developmental age of the plant and changes abruptly with the onset of flowering (Felix and Meins, 1986; Neale *et al.*, 1990). These β-1,3-glucanases also are induced in leaves by viral (Kauffmann *et al.*, 1987; Vögeli-Lange *et al.*, 1988), bacterial or fungal pathogens (Meins and Ahl, 1989), by the stress hormone ethylene (Felix and Meins, 1987), and by ozone (Ernst *et al.*, 1992). In cultured cells, their expression is down-regulated by combinations of the hormones auxin and cytokinin (Felix and Meins, 1986; Mohnen *et al.*, 1985).

To understand better the regulation and physiological function of class I β-1,3-glucanases, we fused the upstream sequence of the tobacco β-1,3-glucanase B (GLB) gene (Sperisen *et al.*, 1991) to the β-glucuronidase (GUS) reporter gene and examined the pattern of chimeric gene expression in transgenic tobacco. Here we show that the GLB promoter is active in a highly localized region of the endosperm during seed germination. The patterns of GUS and β-1,3-glucanase activity found suggest a novel function for β-1,3-glucanases in the germination of dicotyledonous seeds.

Results

Three chimeric genes were constructed and introduced into Havana 425 tobacco by *Agrobacterium*-mediated leaf disc transformation. The GLB–GUS construct contains 1.6 kb of upstream sequence of GLB inserted in front of the GUS and CAT coding sequences (Figure 1). The CAT reporter gene was included as an internal standard for deletion analyses. The promoterless GUS construct pGLB105 served as a negative control and the 35S–GUS construct, containing 528 bp of CaMV 35S promoter sequence (Hart *et al.*, 1993), as a positive control for GUS expression. High-expressing, monogenic lines transformed with the GLB–GUS construct were used for the expression studies presented here.

We verified that the GLB promoter used in GLB–GUS constructs was sufficient for developmental, hormonal, and pathogenesis-related expression typical of class I β-1,3-glucanases. These results are similar to those obtained with another tobacco class I β-1,3-glucanase promoter (van de Rhee *et al.*, 1993) and will be described elsewhere. The present study focuses on GLB–GUS expression during seed germination. Figure 2(a) and (b)

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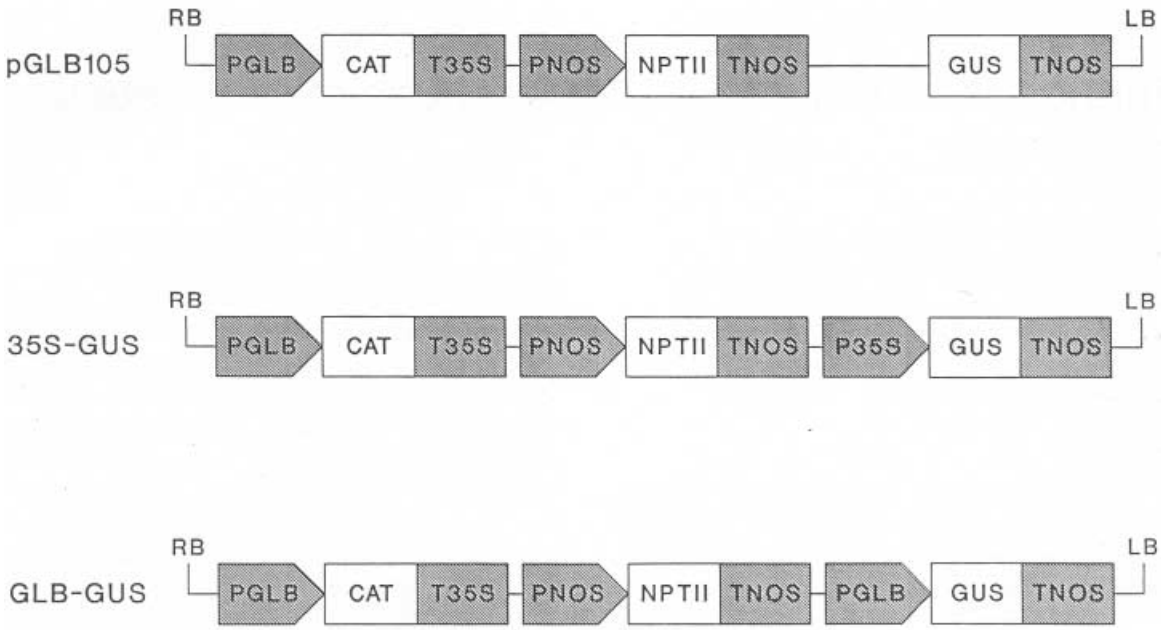

Figure 1. Schematic diagram of the T-DNA region of GUS reporter gene plasmids.
The promoterless construct (pGLB105), the CaMV 35S RNA promoter construct (35S–GUS), and the tobacco class I β-1,3-glucanase B promoter construct (GLB–GUS) are derivatives of pBI101 (Jefferson *et al.*, 1987). Coding sequences (open boxes): CAT, chloramphenicol acetyltransferase; GUS, β-glucuronidase; NPTII, neomycin phosphotransferase. Regulatory sequences (cross-hatched boxes): PGLB, −1630 to +6 of upstream sequence of the tobacco class I β-1,3-glucanase B gene on a 1675 bp *Sal*I/*Bam*HI fragment (Hart *et al.*, 1993); PNOS and TNOS, promoter and terminator of the nopaline synthase gene; P35S and T35S, 528 bp of CaMV 35S upstream sequence on a *Sal*I/*Bam*HI fragment (Hart *et al.*, 1993) and CaMV 35S RNA terminator, respectively. The right (RB) and left (LB) T-DNA borders are indicated.

show germinating seeds stained with X-Gluc 4 days after imbibition. Based on staining, the GLB promoter was active in a specific layer of cells surrounding the emerging seedling. The seedling itself was not stained at this stage of development. A similar pattern of staining was observed when GLB–GUS seedlings and seed coats with adhering endosperm were separated manually after 3 days imbibition but prior to germination (Figure 2c and e). In contrast, both the seedling and most of the seed coat and adhering endosperm of 35S–GUS transformants were stained (Figure 2d and f) indicating that localized expression of GUS at the site where the seedling will emerge depends on the GLB promoter. No staining for GUS activity was observed in germinating seeds from plants transformed with the promoterless GUS construct (data not shown).

To determine whether GUS is expressed in the seed coat or in the endosperm, populations of germinating seeds from homozygous and hemizygous monogenic GLB–GUS transformants were scored for GUS activity. If GUS is expressed in the seed coat, which is of maternal origin, then all of the progeny should be GUS positive. On the other hand, if GUS is expressed in the triploid endosperm, which contains maternal and paternal genes, then the GUS positive phenotype should segregate 3:1. As shown in Table 1, GUS expression segregated 3:1 in seedlings from a hemizygous parent whereas all seedlings from a homozygous parent were GUS positive. Therefore, the GLB promoter is expressed in the endosperm and not in the seed coat. This was confirmed by measuring GUS activity quantitatively in the seed coat and adhering endosperm tissue separated manually from germinating seedlings. The pooled material derived from hemizygous parents gave half the GUS activity of comparable material from homozygous plants as predicted for endosperm-specific expression proportional to gene dose.

β-1,3-Glucanase and the GUS reporter gene driven by the GLB promoter showed similar patterns of expression during germination (Table 1). The activity of both enzymes was very low in dry seeds homozygous for the transgene and increased dramatically during germination. High values of the enzymes were detected 3 days post-imbibition before the endosperm was penetrated by the primary root, and the values continued to increase on day 4 after the endosperm had ruptured (also see Figure 2b and e).

Several isoforms of β-1,3-glucanase have been identified in tobacco: the approximately 36 kDa class II enzymes, PR-2, PR-N and PR-O; the 41 kDa class II stylar enzymes; the approximately 34 kDa class III enzyme PR-Q'; and, the 33 kDa class I enzymes (reviewed by

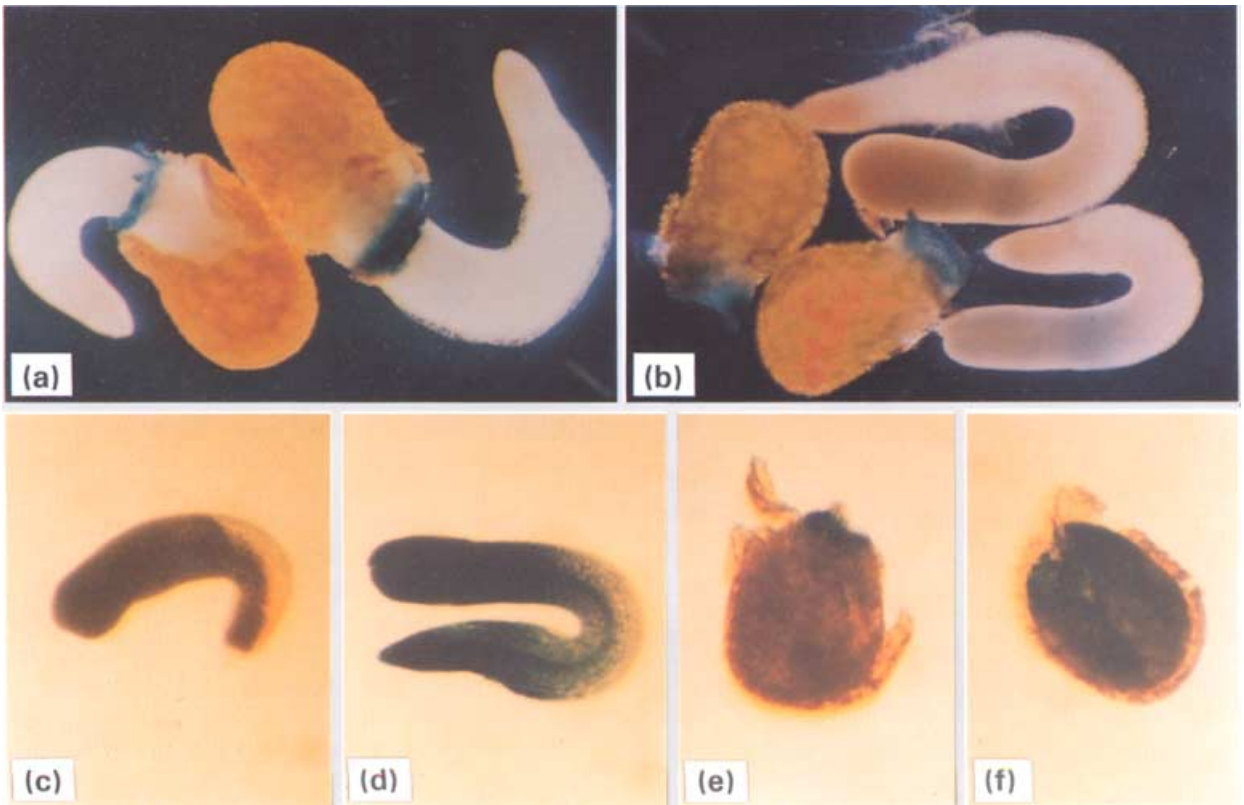

Figure 2. Histochemical location of GUS in germinating transgenic tobacco seeds.
(a) Seeds of a primary transformant containing the GLB–GUS construct 4 days after imbibition.
(b) Same as in (a), but after staining, the embryo and seed coat plus endosperm were manually separated. The faint blue color in the upper embryo is due to leakage from staining of the endosperm.
(c) Embryo dissected from ungerminated GLB–GUS seeds 3 days after imbibition.
(d) Embryo dissected from ungerminated 35S–GUS seeds 3 days after imbibition.
(e) Seed coat and adhering endosperm tissue dissected from ungerminated GLB–GUS seeds 3 days after imbibition.
(f) Seed coat and adhering endosperm tissue dissected from ungerminated 35S–GUS seeds 3 days after imbibition.
Magnification: 40×.

Table 1. β-1,3-Glucanase and GUS activity in seed coat and endosperm tissues of homozygous and hemizygous GLB–GUS transformants

Developmental stage	Genotype of parent plant	Enzyme activity per seed		GUS staining of seeds	
		β-1,3-Glucanase[b]	GUS[c]	GUS positive	Total scored
Dry seed[a]	Homozygous	3.1	2.1	–[d]	–
Germinated, 3 days (endosperm intact)	Homozygous	196	46	–	–
	Hemizygous	138	23	–	–
Germinated, 4 days (endosperm ruptured)	Homozygous	426	265	64	64
	Hemizygous	444	136	43	60

[a]Entire seed.
[b]Activity in pg of class I β-1,3-glucanase equivalents.
[c]fmol MU min^{-1}.
[d]Not determined.

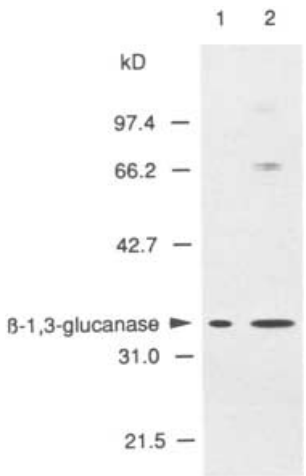

Figure 3. Immunodetection of class I β-1,3-glucanase protein in extracts of germinating seeds.
Purified tobacco class I β-1,3-glucanase (lane 1, 10 ng β-1,3-glucanase) and an extract from manually dissected seed coat plus adhering endosperm tissue from tobacco seeds imbibed for 4 days (lane 2, equivalent in activity to 20 ng of class I β-1,3-glucanase) were fractionated by SDS–PAGE, blotted to nitrocellulose and immunodecorated with an anti-class I β-1,3-glucanase antibody. The position of molecular weight markers and class I β-1,3-glucanase are indicated.

Meins *et al.*, 1992). The isoform expressed in the endosperm was established by immunoblot analysis of extracts with high β-1,3-glucanase activity prepared from seed coat plus endosperm of germinating seeds 4 days after imbibition. The antibody used, which is directed against class I tobacco β-1,3-glucanases, also cross-reacts with the other known tobacco isoforms (Neuhaus *et al.*, 1992; data not shown). A single major band corresponding in size to the authentic 33 kDa class I β-1,3-glucanases of tobacco was found (Figure 3) indicating that the β-1,3-glucanase activity measured during seed germination is primarily due to the class I isoforms.

Discussion

Our findings suggest a novel role for class I β-1,3-glucanases in the germination of dicot seeds. Germinating seedlings from plants transformed with the GLB–GUS construct showed a highly localized pattern of expression of the GUS reporter gene in the endosperm at the site of radicle penetration. When embryo and seed coat were manually dissected and independently stained for GUS, the endosperm stained for GUS activity exclusively at the micropylar end. The embryo itself was never stained (Figure 2b and c). In contrast, in dissected germinating seeds of plants transformed with the 35S–GUS construct, the entire endosperm stained evenly blue and high levels of GUS expression were also observed in cotyledons and primary roots (Figure 2d and f). Thus, the localized expression of the GLB promoter at the site of radicle penetration is specific for the GLB promoter. There is strong evidence that the pattern of expression of the GLB promoter observed reflects the pattern of class I β-1,3-glucanase expression. Western blot analysis of dissected germinated seeds revealed the presence in the seed coat plus endosperm tissue of at least one protein that co-migrates with class I β-1,3-glucanases. Furthermore, β-1,3-glucanase and GUS activities were shown to increase coordinately in the seed coat plus endosperm tissue during germination. The fact that β-1,3-glucanase and the GUS reporter gene driven by the GLB promoter are expressed in the endosperm prior to penetration of the radicle through the endosperm makes it unlikely that transcriptional activation of class I β-1,3-glucanase is due to a wounding response as has been suggested for a bean chalcone synthase promoter (Schmid *et al.*, 1990).

We propose that the physiological significance of class I β-1,3-glucanase in seed germination is digestion of cell wall material to facilitate the protrusion of the radicle through the endosperm. This hypothesis is supported by two observations. In lettuce, which is a dicot, periodic acid-Schiff positive material (presumably polysaccharide) is lost from the endosperm starting before emergence of the radicle (Jones, 1974). Digestion of the endosperm cell wall proceeds from the plasma membrane outward suggesting that endosperm cells produce hydrolytic enzymes that degrade the polysaccharide. In barley, which is a monocot, the walls of the aleurone cells of the endosperm contain β-1,3-glucans, and β-1,3-glucanase activity is strongly induced during imbibition (Taiz and Jones, 1970). Incubation of isolated aleurone layers with GA_3 enhances secretion of β-1,3-glucanase and results in the disappearance of polysaccharides suggesting that β-1,3-glucanase is responsible for the digestion of aleurone walls.

A regulatory sequence, the –300 element, is present in the promoters of many cereal storage protein genes, which are only expressed in the endosperm (Colot *et al.*, 1987; Forde *et al.*, 1985; Matzke *et al.*, 1990). The highly conserved heptanucleotide core of this sequence, TGTAAAG, is also present at a similar location in the promoter of tobacco class I β-1,3-glucanase genes (Sperisen *et al.*, 1991). Reporter gene studies have shown that the temporal and spatial pattern of expression of cereal seed storage proteins is conserved in transgenic tobacco (Colot *et al.*, 1987; Marris *et al.*, 1988; Matzke *et al.*, 1990). Based on deletion analysis and expression in transgenic tobacco it has been proposed that the *cis*-regulatory elements necessary for endosperm-specific expression of monocot genes can be recognized by transcription factors in the dicot tobacco (Colot *et al.*, 1987; Matzke *et al.*, 1990). In contrast to cereal storage proteins, which accumulate in the endo-

sperm during seed maturation, the GLB promoter is activated during seed germination. Thus, if the conserved sequence motif is important for transcriptional activation of the GLB promoter, it probably has a role in spatial rather than temporal regulation of gene expression. Further studies are required to establish the transcriptional role of this sequence during tobacco seed germination.

Experimental procedures

Plasmid construction and plant transformation

The plasmids pGLB105, GLB-GUS and 35S-GUS (Figure 1) are derivatives of pBI101 (Jefferson *et al.*, 1987). pGLB105 was constructed from pBI101 and pGLBP1 (Hart *et al.*, 1993) in two steps: first, a *Xho*I adaptor was inserted into the *Sac*II site close to the right border in the T-DNA of pBI101 to give pXBI. Then, after elimination of the unique *Bam*HI site in pGLBP1 and conversion of the *Sac*I sites to *Sal*I sites, an expression cassette containing 1630 bp of GLB promoter sequence, the CAT coding region and CaMV 35S termination signal was transferred as a *Sal*I fragment from pGLBP1 to the *Xho*I site of pXBI to give pGLB105. The GLB–GUS transcriptional fusion was made by ligating a 1675 bp *Sal*I/*Bam*HI fragment (Hart *et al.*, 1993) containing 1630 bp of GLB promoter sequence into pGLB105 cut with *Sal*I and *Bam*HI. To create the 35S–GUS promoter–reporter gene fusion, a *Sal*I/*Bam*HI fragment containing 528 bp of CaMV 35S promoter sequence (Hart *et al.*, 1993) was cloned in front of the GUS coding sequence of pGLB105. All intermediate plasmids were transformed into *Escherichia coli* strain HB101.

The plasmids pGLB105, GLB–GUS and 35S–GUS were mobilized into the disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) by triparental mating (Hooykaas *et al.*, 1977) using *E. coli* strain HB101 containing pRK2013 (Figurski and Helinski, 1979) as a helper strain. The integrity of the constructs was verified by restriction enzyme analysis. Transgenic *Nicotiana tabacum* cv. Havana 425 plants were generated by the method of Horsch *et al.* (1985) except that *Agrobacterium*-infected leaf pieces were placed adaxial side up on shoot-inducing medium. Plants homozygous for the transgene were obtained from primary transformants showing a monogenic segregation of the kanamycin-resistance trait.

Treatment of transgenic plants

To identify primary transformants with high levels of regulated GUS expression, 0.5 cm diameter leaf discs were prepared from an upper leaf of approximately 5 cm tall, axenically grown plants. Four replicate discs were either frozen directly in liquid nitrogen, or after 7 days culture on LS medium without hormones (Linsmaier and Skoog, 1965) supplemented with 50 μg ml^{-1} kanamycin sulfate, 5 μg ml^{-1} chlorophenol red as pH indicator and 10 g l^{-1} agar. For analysis of gene expression during seed germination, seeds were surface sterilized with 1.4% (w/v) NaOCl, extensively washed in sterile water, and placed on a filter paper on LS medium without hormones supplemented with 400 μg ml^{-1} kanamycin sulfate.

Assays for GUS activity

GUS activity in tissue extracts was assayed by the fluorometric method of Jefferson *et al.* (1987) modified for microtiter plates. In brief, extracts in a final volume of 300 μl in GUS extraction buffer and 1 mM 4-methylumbelliferyl-β-D-glucuronide (Sigma) were incubated at 37°C in black Microfluor plates (Dynatech Laboratories). The reaction was terminated by transferring 50 μl of the reaction mixture to an adjacent well containing 150 μl of 0.24 M Na_2CO_3. Fluorescence was measured at several time points in a microplate reader (Titertek Fluoroscan II, Flow Laboratories). GUS activity is expressed as pmoles of 4-methylumbelliferol (MU) per minute.

To stain for GUS activity, germinating seedlings were mounted on microscope slides, covered with a cover slip and stained with X-Gluc solution consisting of 0.1 M $NaPO_4$, pH 7.0, 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl glucuronide (Research Organics Inc, Cleveland, Ohio) and incubated for 12–24 h at 37°C in a moist chamber. The samples were cleared by several changes in methanol:acetic acid (3:1).

Assays for β-1,3-glucanase

β-1,3-Glucanase activity was measured in tissues by the radiometric assay of Keefe *et al.* (1990) using reduced [^{3}H]-laminarin as a substrate and is expressed in equivalents of the class I enzyme. Immunoblot analysis of β-1,3-glucanases was as described (Keefe *et al.*, 1990) except that the second antibody was coupled to alkaline phosphatase and staining was with 0.033% (w/v) nitro blue tetrazolium, 0.016% (w/v) bromochloroindolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris/HCl, pH 9.5.

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